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Tumor progression induces the growth of endothelial cells by releasing angiogenic factors. This is accompanied by down-regulation of local tissue inhibitors of endothelian cell proliferation such as angiostatin and endostatin. Both proteins target normal endothelial cells and effectively regress large tumors in animals. However, animal studies demonstrate that an effective treatment requires long-term administration of angiogenesis inhibitors. Thus, delivery of angiogenesis inhibitor genes to tumor sites should increase local concentration of these proteins, leading to the retardation of tumor progression and metastasis. During the past fiscal year, we have generated two HIV vectors containing the endostatin and angiostatin cDNA. Human fibroblasts transduced with these vectors proliferated normally whereas the proliferation of primary HUVEC was inhibited by the transduction. For efficient gene expression in prostate cancer cells, we have generated HIV vectors containing the GFP gene controlled by the CMV IE promoter, the LTR from SFFV and the ubiquitin C promoter. We will determine the level of GFP expression from these three promoters in human prostate cancer cell lines.

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Introduction

Tumor progression induces the growth of endothelial cells by releasing angiogenic factors. This is accompanied by down-regulation of local tissue inhibitors of endothelian cell proliferation such as angiostatin and endostatin. Both proteins target normal endothelial cells and effectively regress large tumors in animals. However, animal studies demonstrate that an effective treatment requires long-term administration of angiogenesis inhibitors. Thus, delivery of angiogenesis inhibitor genes to tumor sites should increase local concentration of these proteins, leading to the retardation of tumor progression and metastasis. We propose to use HIV vectors to deliver the endostatin and angiostatin genes into human prostate cancer cell lines in culture. The effect of these two proteins will be evaluated by tumor formation and metastasis in nude mice grafted with the transduced cells. During the past fiscal year, we have generated two HIV vectors containing the endostatin and angiostatin cDNA. Human fibroblasts transduced with these vectors proliferated normally whereas the proliferation of primary HUVEC was inhibited by the transduction. For efficient gene expression in prostate cancer cells, we have generated HIV vectors containing the GFP gene controlled by the CMV IE promoter, the LTR from SFFV and the ubiquitin C promoter. We will determine the level of GFP expression from these three promoters in human prostate cancer cell lines.

BODY

During this fiscal year, we have systematically evaluated an HIV-derived fragment, consisting of a polypurine tract sequence (cPPT) [1, 2], that significantly increased the transduction efficiency of HIV vectors in human hematopoietic progenitor cells and hepatocytes [3, 4]. This fragment was incorporated into all of our HIV vector constructs for more efficient gene delivery into human cells, although its effect on the transduction of human prostate cancer cells remained unknown. We have re-cloned the endostain cDNA into this 3rd-generation HIV vector. In addition, the angiostatin cDNA was also cloned into the new HIV vector as shown in Fig. 1. Both vectors contain the GFP gene and the transduced cells and the transduction efficiency can be monitored by GFP expression. The infectious titers for both vectors were similar, ranging between 10^5 and 10^6 transduction units (TU)/ml when titered on human fibfosarcoma HT1080 cells. Since these vectors were pseudotyped with the VSV-G protein, we were able to further concentrate the vector titer to more than 10^7 TU/ml by ultracentrifugation [3]. We compared the transduction efficiency of the endostatin vector at different multiplicity of infection (moi) in HT1080 cells, primary human umbilical vein endothelial cells (HUVEC) and human prostate cancer PC3 cells. As shown in Fig. 2, at the same moi, the HIV vector transduced HUVEC cells most efficiently whereas the transduction efficiency in PC3 cells was 4-5 fold lower. At moi of 5, only 20% of the PC3 cells were transduced by the vector. This study suggests that high vector titers may be required to efficiently deliver the endostatin and angiostatin cDNAs into human

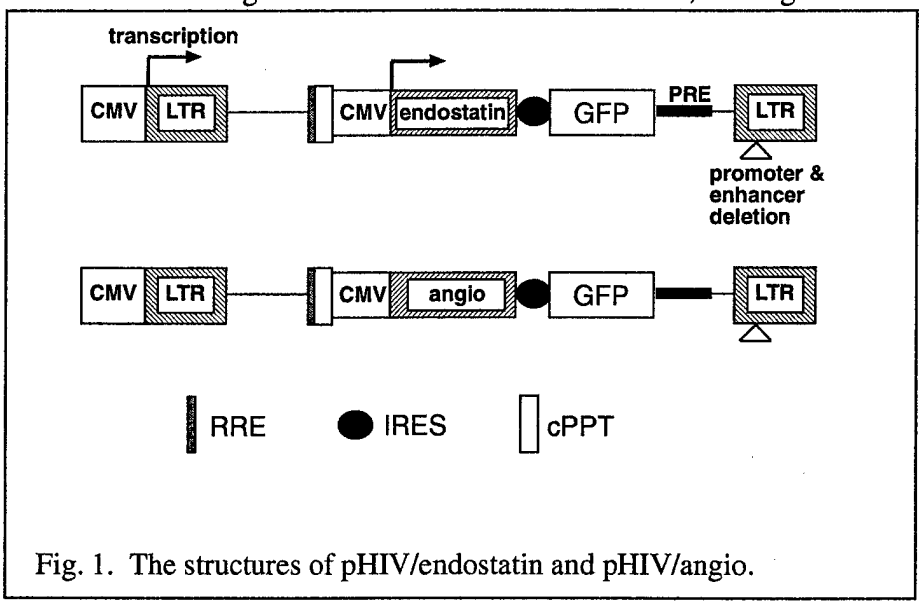

Fig. 1. The structures of pHIV/endostatin and pHIV/angio.

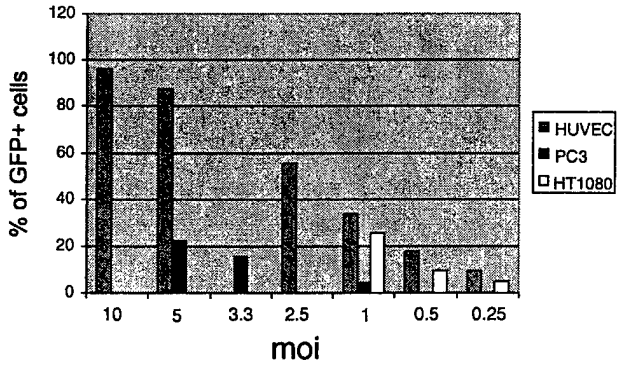

Fig. 2. Comparison of the HIV transduction efficiency in human cells.

prostate cancer cells. To determine whether expression of endostatin and angiostatin has any adverse effect on cell proliferation, HT1080 cells were transduced with the two vectors either alone or together at various moi and cell proliferation was monitored with the MTT assay. As shown in Fig. 3, transduction by these vectors had little effect on cell proliferation. Although HIV/angio transduction led to decreased cell proliferation at moi of 2, this effect was probably non-specific since transduction with both vectors at the same moi did not significantly alter cell proliferation (Fig. 3, endo-angio). Thus, expression of angiogenesis, as expected, does not alter the growth of a non-endothelial cell origin. In contrast, when primary HUVEC were transduced with the vectors, cell proliferation was inhibited by HIV7/endo and HIV7/angio transduction (Fig. 4). This result indicates that expression of angiostatin and endostatin can act on the proper target and specifically inhibit cell proliferation. Prostate cancer PC3 cells were transduced with HIV-GFP, HIV/endo, HIV/angio and HIV/endo plus HIV/angio at moi of 5. We are currently assessing the transduction efficiency by determining the percentage of GFP+ cells in each cell population. In the case that not all the cells are transduced with the vector, we will perform repeated transduction until more than 95% of the cells become GFP+. The effect of angiogenesis inhibitor expression will then be evaluated by transplanting the cells onto nude mice for tumor formation. The relatively low transduction efficiency of HIV vectors in prostate cancer cells (Fig. 2) further emphasizes the importance of testing strong promoters for efficient gene expression in prostate cancer cells. During the past year, we have compared the promoter strength of the CMV IE promoter and the SF promoter derived from the long terminal repeat (LTR) of spleen focus forming virus (SFFV) in different cell types [3]. These promoters functioned in the context of an HIV vector and drove the expression of the GFP gene. In HT1080 cells, the CMV IE promoter was a much stronger promoter whereas the SF LTR functioned more efficiently in human hematopoietic cells [5]. We have also obtained the human ubiquitin C promoter which directs high-level gene expression in most of the

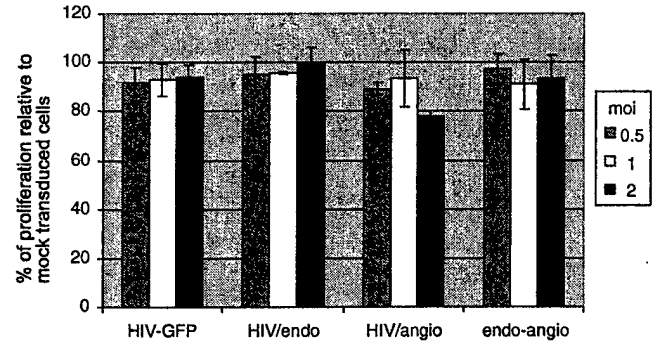

Fig. 3. The effect of HIV/endo and HIV/angio transduction on HT1080 proliferation.

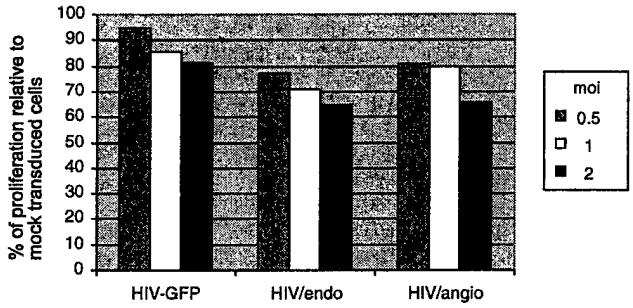

Fig. 4. Expression of endostatin and angiostatin inhibits HUVEC proliferation.

mammalian cells. This promoter has been inserted into our HIV vector for GFP expression. We are currently comparing the level of GFP gene expression from the three promoters in PC3 cells. The most efficient promoter will then be used to drive the expression of the endostatin and angiostatin cDNAs.

Key Research Accomplishments

1. Complete the construction of 3rd generation HIV vectors containing the endostatin and angiostatin cDNAs

2. Demonstrate the inhibition of endothelial cell proliferation when transduced with the two HIV vectors

3. Complete the construction of three HIV vectors containing different promoters to drive GFP gene expression in prostate cancer cell lines

Reportable Outcomes

1. Construct two 3rd generation HIV vectors containing the endostatin and angiostatin cDNAs that can be applied to the treatment of multiple tumor cell types

2. Claudia Kowolik, a Postdoctoral Fellow, received training in HIV vector construction and production

Conclusion

1. High-titer HIV vectors (10^5-10^6 TU/ml) containing the endostatin and angiostatin cDNAs could be obtained. They could be further concentrated by ultracentrifugation.

2. These vectors could transduce human cells with different efficiency. The transduction efficiency in prostate cancer cell lines, however, was lower than that in other cell types.

3. Transduction with these vectors did not alter the proliferation of HT1080 cells but retard the proliferation of primary HUVEC, suggesting that the two proteins acted on the proper cell type (endothelial cells).

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Appendices

1. Yam PY, Li S, Wu J, Hu J, Zaia JA, Yee JK. **Design of HIV Vectors for Efficient Gene Delivery into Human Hematopoietic Cells.** *Mol Ther* 2002,5:479-484.

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METHOD

Design of HIV Vectors for Efficient Gene Delivery into Human Hematopoietic Cells

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Vectors derived from human immunodeficiency virus (HIV) hold promise for efficient gene delivery into human hematopoietic cells. In this study, HIV vectors containing different combinations of *cis*-acting elements, including the HIV central flap sequence, and the woodchuck posttranscriptional regulatory element (WPRE) in combination with two different promoters, were used to transduce primary human lymphocytes and cord blood CD34+ progenitor cells. The effect of these elements on the transduction efficiency and transgene expression was systematically evaluated. The results demonstrate that with the combination of flap, WPRE sequences, and the promoter derived from spleen focus-forming virus (SFFV), a foreign gene can be efficiently delivered into primary human T lymphocytes and cord blood CD34+ cells. The study establishes the parameters for proper vector design to efficiently deliver foreign genes into human hematopoietic cells.

Key Words: HIV vectors, lymphocytes, CD34+ cells, transduction, gene expression

INTRODUCTION

The ability to deliver foreign genes into hematopoietic cells provides the opportunity to treat genetic or acquired diseases in human patients. Peripheral T lymphocytes and hematopoietic stem cells (HSCs) are particularly useful targets for genetic modification. T lymphocytes are easy to purify and can proliferate vigorously in culture with appropriate cytokine stimulation; moreover, the lifespan of some lymphocyte subpopulations can persist *in vivo* for up to several years [1–3], making them ideal targets for long-term transgene expression. HSCs have the properties of self-renewal and differentiation into cells of all blood lineages. Successful gene delivery into HSCs can therefore permanently correct a defect in the hematopoietic system. So far, the most widely used vectors for delivering genes into HSCs are retroviral vectors derived from murine leukemia virus (MLV). However, the low transduction efficiency and poor long-term gene expression in these cells have hampered their clinical application [4–6].

Vectors derived from lentiviruses hold promise to overcome the problems encountered with the MLV vectors. Lentiviruses are known to infect nondividing cells such as macrophages because of the presence of the nuclear localization signal in several virally encoded proteins [7–9]. Vectors derived from HIV-1 were shown to transduce CD34+ hematopoietic progenitor cells isolated from human bone marrow and cord blood [10–16]. The transduced progenitor cells have long-term engraftment ability in nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice and can differentiate into cells of both myeloid and lymphoid lineages *in vivo*. These results demonstrate the potential of HIV-based vectors to deliver genes into primitive hematopoietic cells for potential treatment of human diseases.

In the current study, we have systematically evaluated two *cis*-acting elements for their effect on HIV vectors to transduce primary human T lymphocytes and CD34+ cells. One sequence element, termed central DNA flap, consists of a polypurine tract sequence (cPPT) and a central termination sequence (CTS). It is present within the HIV polymerase (pol) gene and is able to facilitate nuclear import of the viral preintegration complex [17,18]. A second sequence element, termed WPRE, present in the genome of woodchuck hepatitis virus, was shown to increase transgene expression in the context of plasmid DNAs or viral vectors [19–21]. In this study, we also compare the activity of two promoters for transgene expression in hematopoietic cells: the promoter derived from the immediate early (IE) gene of cytomegalovirus (CMV) and the promoter derived from the long terminal repeat (LTR) of SFFV virus [22]. The CMV promoter functions strongly in cells such as fibroblasts, but its promoter activity is significantly diminished in hematopoietic cells. In contrast, efficient transcription from the SFFV LTR was observed not only in fibroblasts but also in several cell types of the myeloid lineage [22].

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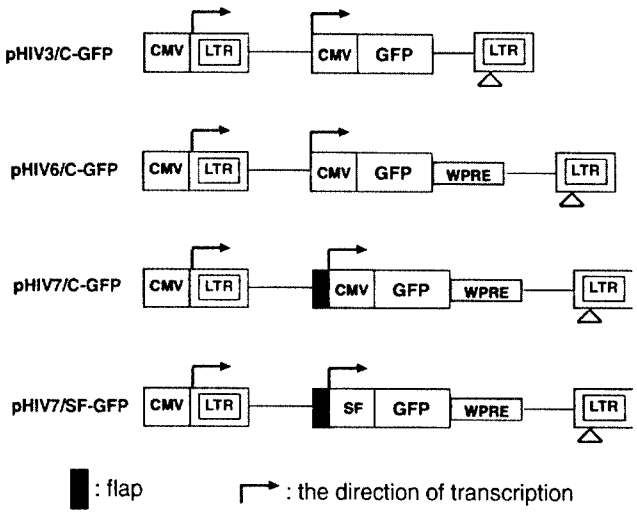

FIG. 1. Schematic representation of HIV-based SIN vectors. Δ represents a 400-bp deletion in the 3′-LTR that completely removes the HIV enhancer and promoter sequences. All vectors contain the enhanced GFP gene as the reporter. Arrows depict the direction of transcription. The 5′-LTR of each vector construct contains a fusion promoter with the CMV enhancer linked to the promoter of the HIV LTR. The cis-regulatory elements used to drive GFP expression include the IE promoter and enhancer of cytomegalovirus (CMV) and the LTR of spleen focus-forming virus (SF). The solid boxes represent the 190bp flap sequence from HIV-1.

Our results demonstrated that the central flap element, when combined with the WPRE sequence and the SFFV LTR in a self-inactivating (SIN) vector backbone, facilitated efficient transduction and transgene expression in primary human T lymphocytes and HSCs.

While the CMV IE promoter was relatively inefficient in CD34+ cells and their differentiated progenies such as erythrocytes and myelomonocytic cells, its activity was upregulated in differentiated dendritic cells. These studies optimize the parameters for proper design of efficient HIV vectors to transduce primary human hematopoietic cells in culture and should be useful for future somatic gene therapy applications.

RESULTS

Generation of HIV Vectors

To determine the parameters in vector design for efficient transduction of primary human T lymphocytes and HSCs, a set of four HIV vectors were constructed (Fig. 1). HIV3/C-GFP contains the backbone of an HIV vector with the green fluorescence protein (GFP) gene controlled by the CMV IE promoter. The HIV enhancer in the U3 region of the 5′-LTR was replaced with the CMV IE enhancer, enabling the production of this vector in the absence of the Tat protein [23]. The HIV enhancer and promoter sequences in the U3 region of the 3′-LTR were removed, resulting in the production of a SIN vector [24]. HIV6/C-GFP is similar to pHIV3/C-GFP except that the WPRE sequence was inserted immediately downstream of the GFP gene. Insertion of the HIV central DNA flap sequence upstream of the CMV promoter in pHIV6/C-GFP created pHIV7/C-GFP. To compare different promoters, the CMV promoter in pHIV7/C-GFP was replaced with the SFFV LTR to create pHIV7/SF-GFP. Infectious vectors were generated from 293T cells, and the titer of each vector was determined in HT1080 cells, a human fibrosarcoma line, and was generally in the range between 10^6 and 10^7 transduction units/ml.

Transduction of Primary Human T Lymphocytes

Primary human T lymphocytes were isolated from normal donor's peripheral blood. The cells were expanded using anti-CD3 and anti-CD28 antibodies, followed by vector transduction at a multiplicity of infection (MOI) of 30. Flow-cytometric analysis of GFP+ cells in one of the transduced donor samples (donor 2) before normalization to the amount of the input p24 is shown in Fig. 2. Table 1 summarizes the transduction of T lymphocytes isolated from five different donors. The percentage of HIV6/C-GFP-transduced cells, when normalized to the amount of p24, was not significantly different from that of HIV3/C-GFP-transduced cells (t-test, $P = 0.3$). In contrast, the mean fluorescence intensity of the HIV6/C-GFP-transduced cells was consistently higher (between 1.6- and 3.1-fold, $P = 0.05$) than that of the HIV3/C-GFP-transduced cells, irrespective of the transduction efficiency (Table 1). Thus, the WPRE sequence increased transgene expression from the CMV promoter but had little effect on the transduction efficiency in primary human T lymphocytes. Inclusion of the flap sequence in HIV7/C-GFP enhanced the transduction efficiency by ~ 4- to 10-fold when compared with HIV6/C-GFP ($P = 0.02$), and a slight increase in mean fluorescence intensity was also observed (Table 1). It is unlikely that this increase in the transduction efficiency is the result of

TABLE 1: Transduction of primary T lymphocytes with HIV vectors

	% of transduction/μg of p24 (MFI)[a]			
Donor[b]	HIV3/C-GFP	HIV6/C-GFP	HIV7/C-GFP	HIV7/SF-GFP
1	3.0 (20)	9.7 (43)	34.6 (81)	19.5 (127)
2	1.5 (72)	2.8 (128)	31.2 (209)	32.3 (546)
3	2.0 (58)	1.2 (91)	8.9 (80)	13.9 (219)
4	2.9 (49)	1.6 (88)	16.9 (131)	15.0 (194)
5	1.8 (14)	0.6 (43)	5.6 (83)	9.0 (216)
				1.0 (71)[c]

[a]% of transduction represents the fraction of GFP+ cells in total T lymphocytes 5 days after transduction. These values were normalized to the amount of the input p24. MFI, mean fluorescence intensity.
[b]Primary human T lymphocytes were isolated from 5 different donors and transduced with the vector indicated.
[c]The transduced T lymphocytes were treated with 5 mM of AZT during incubation.

pseudotransduction, because addition of 3′-azido-3′ deoxythymidine (AZT) caused a 90% inhibition in transduction (Table 1, donor 5). The presence of the flap sequence in an HIV vector thus significantly increased its transduction efficiency in primary human T lymphocytes.

Although the CMV IE promoter functions strongly in fibroblasts, its activity in hematopoietic cells is relatively inefficient. To identify a more efficient promoter, we compared the activity of the SFFV LTR and the CMV promoter in primary human T lymphocytes. While the transduction efficiency of HIV7/C-GFP and HIV7/SF-GFP in primary lymphocytes was similar, HIV7/SF-GFP-transduced cells demonstrated higher levels of mean fluorescence intensity of GFP than HIV7/C-GFP-transduced cells (Table 1). Lymphocytes from two donors (donors 3 and 5) exhibited almost three-fold difference in the mean fluorescence intensity when transduced by the two vectors (Table 1). Taken together, these results demonstrate that the WPRE and flap sequences in conjunction with an efficient promoter such as the SFFV LTR can significantly increase the transduction efficiency of HIV vectors and transgene expression in primary human T lymphocytes.

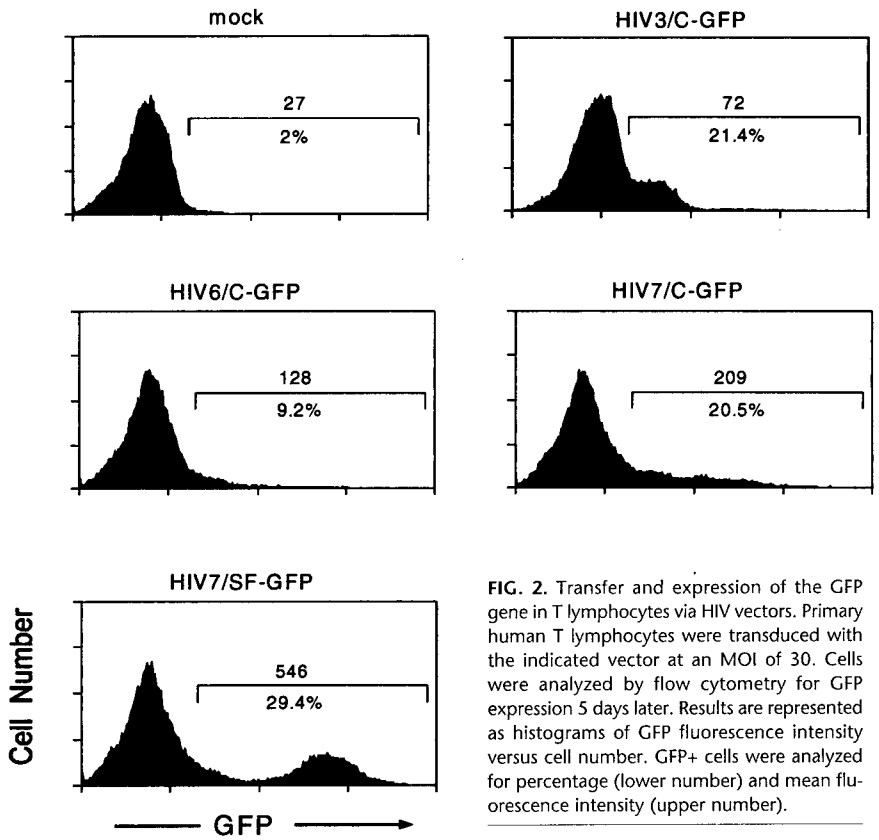

FIG. 2. Transfer and expression of the GFP gene in T lymphocytes via HIV vectors. Primary human T lymphocytes were transduced with the indicated vector at an MOI of 30. Cells were analyzed by flow cytometry for GFP expression 5 days later. Results are represented as histograms of GFP fluorescence intensity versus cell number. GFP+ cells were analyzed for percentage (lower number) and mean fluorescence intensity (upper number).

TABLE 2: Transduction of cord blood CD34+ cells with HIV vectors

	% of transduction/μg of p24 (MFI)		
Donor[a]	HIV6/C-GFP	HIV7/C-GFP	HIV7/SF-GFP
1	1 (50)	13 (90)	ND[b]
2	6 (236)	55 (585)	47 (1434)
3	3 (196)	62 (270)	65 (667)
4	8 (434)	81 (651)	71 (2420)
5	7 (40)	69 (81)	69 (1310)
6	10 (325)	68 (366)	63 (2061)
7	12 (521)	88 (860)	78 (1825)
8	5 (138)	43 (164)	55 (823)

[a]Cord blood CD34+ cells were isolated from 8 donors and were transduced with the vector indicated. % of transduction represents the fraction of GFP+ cells in total CD34+ cells 5 days after transduction. MFI, mean fluorescence intensity.
[b]ND, not done

Transduction of Cord Blood CD34+ Cells

Cord blood CD34+ cells were grown in serum-free culture media in the presence of stem-cell factor (SCF), thrombopoietin (TPO), and Flt3/Flk2 ligand (FL). Preliminary studies demonstrated that such conditions minimized cell proliferation and preserved up to 90% of the CD34+ phenotype for at least 72 hours (data not shown). We then compared the transduction efficiency of cord blood CD34+ cells using HIV6/C-GFP, HIV7/C-GFP, and HIV7/SF-GFP in the culture condition. The results of transducing cord blood CD34+ cells from eight different donors were summarized in Table 2. The transduction efficiency of HIV7/C-GFP, when normalized to the amount of the input p24, was on average 10-fold higher than that of HIV6/C-GFP ($P < 0.001$). Similar results were obtained from the analysis of methylcellulose colonies derived from the transduced cells (data not shown). The mean fluorescence intensity of the HIV7/C-GFP-transduced cells also increased slightly relative to that of HIV6/C-GFP-transduced cells. More efficient migration of the HIV pre-integration complex into the nucleus of HIV7/C-GFP-transduced cells may account for this increase in fluorescence intensity. While the transduction efficiency of HIV7/C-GFP and HIV7/SF-GFP was similar in CD34+ cells, the GFP mean fluorescence intensity from cells transduced with HIV7/SF-GFP was on average five-fold higher than that from cells transduced with

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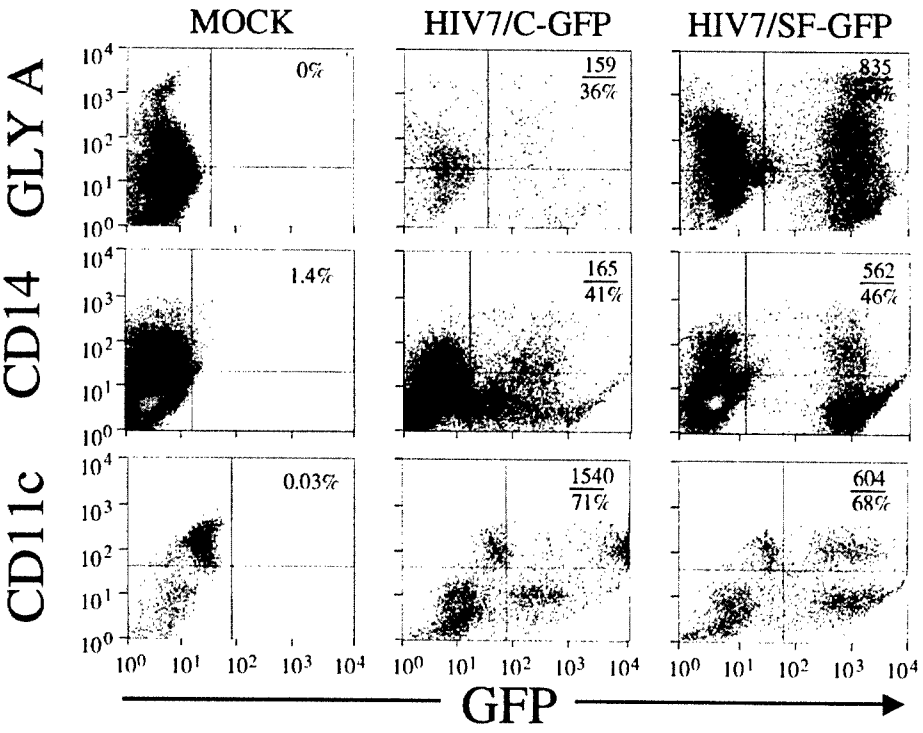

FIG. 3. GFP expression in hematopoietic cell lineages differentiated from transduced cord blood CD34+ cells. The cells were mock-transduced, or transduced with either HIV7/C-GFP or HIV7/SF-GFP at an MOI of 40. The transduced cells were then subjected to the treatment of different cytokines for differentiation into various cell lineages. After differentiation, the cells were analyzed by flow cytometry for the expression of both GFP and lineage-specific markers (glycophorin A, CD14, and CD11c for erythroid, myelomonocytic, and dendritic cells, respectively). The percentage value (lower number) represents the fraction of GFP+ cells in glycophorin A+, CD14+, or CD11c+ cell population, and the mean fluorescence intensity of these differentiated GFP+ cells is also indicated (upper number). Quadrants were set according to staining with isotype-matched negative control antibodies.

HIV7/C-GFP ($P = 0.001$). In one of the cord blood samples (donor 5), HIV7/SF-GFP transduction led to a 16-fold higher GFP expression than HIV7/C-GFP transduction. We concluded from these results that the flap sequence facilitated HIV vector transduction of HSCs in the absence of cytokine-induced cell proliferation, and that the SFFV LTR served as an efficient promoter for transgene expression in these CD34+ cells.

Influence of Differentiation on GFP Expression in Transduced CD34+ Cells

To determine whether the two promoters continued to function in transduced CD34+ cells after differentiation, we subjected the transduced cells to cytokine treatment to allow differentiation into various hematopoietic cell lineages. For erythroid differentiation, a significant fraction of glycophorin A-positive (glyA+) cells continued to express GFP, and the mean fluorescence intensity of GFP in the HIV7/SF-GFP-transduced, glyA+ cells was five-fold higher than that in HIV7/C-GFP-transduced cells (Fig. 3). GFP expression persisted upon differentiation of CD34+ cells into CD14+ myelomonocytic cells, and the expression level in HIV7/SF-GFP-transduced, CD14+ cell population was 3.5-fold higher than that in HIV7/C-GFP-transduced cells (Fig. 3). In contrast, GFP expression in HIV7/C-GFP-transduced, CD11c+ dendritic cells was 2.5-fold more efficient than that in HIV7/SF-GFP-transduced cells (Fig. 3). Similar results were also obtained in the differentiated CD1a+ cell population (data not shown). These results suggest that these two promoters continue to function throughout CD34+ cell differentiation. In differentiated erythroid and myelomonocytic cells, the SFFV LTR functions more efficiently than the CMV IE promoter in driving transgene expression. In contrast, the CMV IE promoter acts as a more efficient promoter in differentiated dendritic cells. Thus, the promoter activity in an HIV vector can be differentially regulated. This regulation appears to be dependent on the differentiation status of the hematopoietic cell lineage.

DISCUSSION

In this study, we systematically compared the effect of the WPRE and flap sequences on transduction efficiency and gene expression of HIV vectors in primary human T lymphocytes and CD34+ cells. Our results demonstrated that the presence of the WPRE sequence had little effect on the transduction efficiency in T lymphocytes but did increase GFP expression. This increase in GFP expression from the CMV IE promoter was modest, between two- and three-fold, and was consistent with the study reported by Ramezani *et al.* [12]. While the effect of the WPRE sequence on the SFFV LTR was not investigated in our study, Ramezani *et al.* reported consistent stimulation of the LTR derived from various MLV family members [12]. Thus, the presence of WPRE would be predicted to facilitate more efficient gene expression from the SFFV LTR. Insertion of the flap sequence significantly increased the transduction efficiency in T lymphocytes. A comparison between HIV7/SF-GFP and HIV3/C-GFP demonstrated that

the average improvement in transduction efficiency was approximately ninefold and the average improvement in GFP expression was approximately sevenfold. Thus, such a combination of various *cis*-regulatory elements significantly boosts the ability of an HIV vector to transduce and express a transgene in primary human T lymphocytes.

In the current study, we used unstimulated cord blood CD34+ cells to evaluate the effect of these elements on transduction and gene expression. This strategy likely mimics the actual transduction conditions used in a gene therapy setting, in that cytokine stimulation of hematopoietic progenitor cells in culture can lead to cell commitment and differentiation. Our studies demonstrated that, under these conditions, cord blood CD34+ cells were susceptible to HIV vector transduction, and up to 70% of the cells exhibited GFP expression. Similar to T-lymphocyte transduction, the flap sequence enhanced the transduction efficiency in CD34+ cells, and the level of enhancement was similar in these two cell populations. In this study, T lymphocytes were stimulated to proliferate while CD34+ cells remained relatively quiescent before the application of HIV vectors. Thus, the presence of the flap sequence enhanced the transduction irrespective of the status of cell proliferation.

For efficient transgene expression in hematopoietic progenitor cells, we compared the activities of the CMV IE promoter and the SFFV LTR. The SFFV LTR was tested because it had been shown to have high promoter activity in both multipotent and lineage-committed myeloid cell lines [22]. Our studies demonstrated that both promoters continued to function after differentiation of the transduced CD34+ cells into different cell lineages in cell culture, suggesting that the differentiation process did not silence the promoter activity in the context of an HIV vector. While GFP expression from the SFFV LTR was consistently higher than that from the CMV IE promoter in myelomonocytic and erythroid cell lineages, the expression from the CMV IE promoter was more efficient in the dendritic cell lineage. Thus, the process of hematopoietic cell differentiation can alter the activity of a promoter inserted into an HIV vector.

Besides modifications of the HIV-based vectors by the incorporation of the WPRE and flap sequences, the HIV promoter and enhancer sequences in the U3 region of the LTR were also removed from the vectors to generate the SIN vector [24,25]. Because the HIV LTR is transcriptionally inactive, the possibility of activating cellular genes flanking the vector integration site would be minimized by this alteration. Moreover, the use of the SIN vector renders it unlikely that the vector could be rescued by a replication-competent HIV and spread to untransduced cells. Because modifications created in the SIN vector have little effect on vector titers [24,25], such vectors would be ideal to deliver a transgene into the hematopoietic system for disease treatment. Our results demonstrate that with the combination of WPRE, flap, and the SFFV LTR, a foreign gene can be efficiently delivered into primary human T lymphocytes and cord blood CD34+ cells. Such vectors should aid in high-level and long-term gene expression in the human hematopoietic system and in the genetic treatment of diseases associated with this system.

MATERIALS AND METHODS

Cell lines. Both 293T and HT1080 cells used in this study were maintained in high-glucose (4.5 g/liter) Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 2 mM L-glutamine, and 100 mg of gentamicin/liter.

Cytokines. The following cytokines were purchased from R&D (Minneapolis, MN): interleukin 2 (IL-2), IL-3, GM-CSF, SCF, FL, TPO, tumor necrosis factor (TNF)-α, and IL-4. Erythropoietin (EPO) was purchased from Amgen (Thousand Oaks, CA).

Plasmid construction and vector production. The construction of pHIV3 has been described [26]. To generate pHIV3/C-GFP, a 1.3-kb fragment containing the gene encoding GFP under the control of the CMV IE promoter was obtained by PCR of pEGFP-C1 (Clontech, Palo Alto, CA) and inserted into the unique *Bam*HI site in pHIV3. To generate pHIV6, a 600-bp *Bam*HI-*Sal*I fragment containing the WPRE sequence was inserted into pHIV3 immediately downstream of the unique *Bam*HI site. The same CMV-GFP cassette described above was then inserted into the unique *Bam*HI site in pHIV6 to generate pHIV6/C-GFP. To generate pHIV7, a 190-bp *Bgl*II-*Bam*HI fragment containing the flap sequence was amplified from pCMV-HIV-1 [27] by PCR and inserted into pHIV6 between the RRE sequence and the unique *Bam*HI site. The CMV-GFP cassette was then inserted into the *Bam*HI site in pHIV7 to generate pHIV7/C-GFP. To generate pHIV7/SF-GFP, the 740-bp fragment containing the GFP gene from pEGFP-C1 was first linked with the 701-bp *Bam*HI-*Kpn*I fragment containing the SFFV LTR [22]. The 1.4-kb fragment containing the GFP gene under the control of the SFFV LTR was then inserted into the *Bam*HI site in pHIV7 to generate pHIV7/SF-GFP.

To produce infectious vectors, 293T cells at a density of 4×10^6 per 10-cm culture dish were transfected with 10mg of pCMV-G, 10 μg of pCMV-HIV-1, and 20 μg of each vector construct by the method of calcium phosphate coprecipitation. Vectors were harvested at 24 and 36 hours after transfection and the titer was determined in HT1080 cells by flow cytometry analysis of GFP expression.

Transduction of primary T lymphocytes. Mononuclear cells (MNCs) were purified by Ficoll-Paque gradient (Pharmacia, Piscataway, NJ). The isolated MNCs were grown in RPMI 1640 medium supplemented with 10% FBS, and the culture dish was pretreated with antibodies directed against CD3 and CD28 cells (BD PharMingen, San Diego, CA) at a concentration of 0.1 μg/ml to stimulate lymphocyte proliferation. Two days after stimulation, the cells were transduced with HIV vectors at an MOI of 30 for 4 hours in a culture dish pretreated with 50 μg/ml Retronectin (Biowhittaker, Walkersville, MD). The transduced cells were pelleted, washed, and resuspended in anti-CD3/anti-CD28-coated culture dishes containing RPMI 1640 medium supplemented with 10% FBS and 5 U/ml IL-2. Five days after transduction, GFP+ cells were analyzed by flow cytometry.

Transduction of CD34+ cells. CD34+ cells were isolated from umbilical cord blood samples. CD34+ progenitor cells were enriched from MNCs by immunomagnetic beads and monoclonal antibody directed against CD34 (Miltenyi Biotech, Auburn, CA). After two cycles of selection, the purity was estimated to be > 90% by fluorescence-activated cell sorting (FACS). For transduction, 4×10^4–1×10^5 CD34+ cells were seeded in 48-well fibronectin-treated plates in 100 μl of BIT9500 medium (Stem Cell Technology Inc., Vancouver, BC, Canada) supplemented with FL, SCF, and TPO at a ratio of 50:50:10 ng/ml. After the cells were incubated overnight at 37°C in 5% CO_2, they were transduced with a single exposure of virus at an MOI of 40. On day 5 after transduction, the cells were analyzed for GFP expression by flow cytometry.

In vitro differentiation of CD34+ cells. For CD34+ cell differentiation into the erythroid lineage, the transduced CD34+ cells were cultured in 15%

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BIT9500 medium supplemented with EPO at 2 U/ml and SCF at 100 ng/ml. Flow cytometry analysis using a monoclonal antibody to glycophorin A was done weekly for 3 weeks to monitor erythroid differentiation. For CD34+ cell differentiation into the myelomonocytic lineage, the transduced CD34+ cells were cultured in 15% BIT9500 medium supplemented with GM-CSF and SCF at 20 ng/ml for 3 weeks. Myelomonocytic differentiation was monitored by flow cytometry analysis using a CD14-specific monoclonal antibody. For CD34+ cell differentiation into the dendritic lineage, the transduced CD34+ cells were cultured in 15% BIT9500 medium supplemented with TNF-α, GM-CSF, and SCF at a ratio of 50 U:200 U:50 ng/ml. After 5 days, IL-4 was added at a concentration of 50 ng/ml, and dendritic differentiation was monitored 12 days later by flow cytometry analysis using monoclonal antibodies specific to CD1a and CD11c. All phycoerythrin-conjugated monoclonal antibodies were purchased from BD PharMingen.

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Preferential Transduction of Human Hepatocytes with Lentiviral Vectors Pseudotyped by Sendai Virus F Protein

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One of the major challenges facing gene therapy is the development of vectors targeting specific cell types. Restricting gene delivery to the relevant cell type leads to reduced T-cell responses to transgene products and prolonged gene expression. In this study, we demonstrate that vectors derived from human immunodeficiency virus (HIV) can be pseudotyped with Sendai virus fusion protein F. Such vectors transduced human hepatoma cells and primary human hepatocytes efficiently, but not non-liver cells. Several different approaches were also taken to significantly increase the titer of the pseudotyped vector. These studies may facilitate HIV vector-mediated gene delivery into liver *in vivo*.

Key Words: lentiviral vector, Sendai virus, hepatocyte

INTRODUCTION

Lentiviral vectors are promising tools for gene therapy studies. Unlike murine leukemia virus (MLV)-based vectors, lentiviruses are able to efficiently transduce proliferating as well as quiescent cells [1–5]. One of the major challenges facing lentiviral-based gene delivery systems is the development of vectors targeting specific cell types. Ubiquitous expression of transgenes, especially in antigen presenting cells, can induce immune responses against vectors or transgene products. This can lead to selective elimination of the transduced cells and limit the application of gene therapy for treatment of chronic disorders. Infection of dendritic cells by adenoviral vectors and adeno-associated viral vectors led to the development of cytotoxic T-lymphocyte responses to the transgene product [6,7]. A previous report [8] demonstrated that restricting the expression of transgenes to skeletal muscle cells led to much weaker $CD8^+$ T-cell responses towards strongly immunogenic transgene products and prolonged transgene expression *in vivo*. These studies emphasize the importance of restricted transgene expression for the successful application of gene therapy to treat human diseases that require prolonged expression of the transgene.

Strategies that direct gene delivery to specific cell types include chemical modification of viral envelope (env) proteins, the use of antibodies to bridge viral env proteins with specific cell-surface molecules, and the use of recombinant env proteins containing cell-specific ligands or single-chain antibodies [9–12]. Formation of stable pseudotypes between HIV particles and viral env proteins has been described in several cases, where the natural $CD4^+$ tropism of HIV has been altered to broaden the host cell range [13,14]. But so far no lentiviral vector has been targeted to deliver genes to a specific cell type.

The Sendai virus F env protein (SV-F) is capable of binding specifically to the hepatic asialoglycoprotein receptor (ASGP-R), mediating the fusion of the viral envelope with the cell membrane [15–17]. MLV vectors pseudotyped with SV-F can specifically transduce human hepatoma cells [18]. To determine whether SV-F could similarly pseudotype HIV particles and achieve liver-specific transduction, we tested the ability of HIV vectors to incorporate SV-F and transduce hepatocytes specifically. As the reported titers of SV-F-pseudotyped MLV vectors were relatively low [18], we further investigated several approaches, including modification of SV-F, the vector, and the transduction method, in an attempt to increase hepatocyte transduction efficiencies with the pseudotyped vectors. Our results suggest that pseudotyping with the SV-F protein has the potential to confer hepatocyte specificity on lentiviral vectors.

RESULTS

Pseudotype Formation between an HIV Vector and SV-F

To determine whether SV-F can be incorporated into HIV particles, we generated HIV-3/β-gal vectors (Fig. 1) pseudotyped with SV-F by transient transfection. HIV-3/β-gal vectors pseudotyped with VSV-G were used as a control. After activation of SV-F with acetylated trypsin, we determined the vector titers on HT1080 cells, a human fibrosarcoma line, and HepG2 cells, a well-differentiated human

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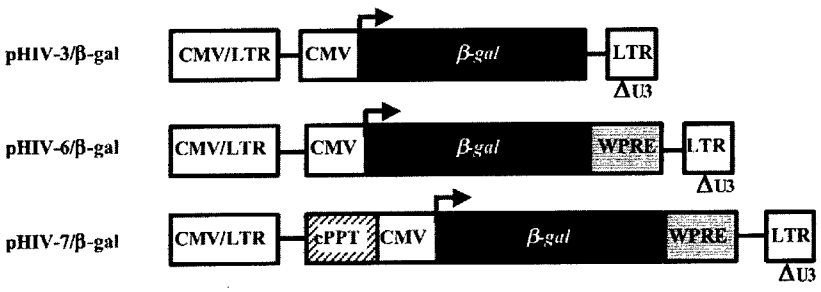

FIG. 1. Structures of the HIV vectors. CMV/LTR represents the fusion between the CMV enhancer of the IE gene and the promoter of the HIV 5′ LTR, in which the HIV sequence upstream of the TATA box was replaced by the CMV enhancer. This modification renders the generation of HIV vectors Tat-independent [46]. CMV represents the 750-bp CMV IE promoter and enhancer sequences. WPRE represents the 600-bp post-transcriptional regulatory element from woodchuck hepatitis virus. cPPT represents the 190-bp central polypurine tract sequence from HIV-1. ΔU3 symbolizes a 400-bp deletion in the 3′ LTR that completely removed the enhancer and promoter sequences in the U3 region of the HIV 3′ LTR. This deletion leads to the production of self-inactivating (SIN) HIV vectors [44]. The arrows indicate the direction of transcription from the internal CMV promoter.

hepatoma line. Cells were stained for β-galactosidase (β-gal) expression 48 hours after transduction. The titer of HIV-3/β-gal(G) in HepG2 cells was 2.6×10^5 transduction units (TU)/ml on average, whereas the titer of HT1080 was in the range of 1.3×10^6 TU/ml (Table 1). In contrast, HIV-3/β-gal(F) was able to transduce HepG2 cells, generating a titer of 6.5×10^3 TU/ml, but not HT1080 cells (Table 1). Despite the similar amounts of vectors used for transduction, as determined by the p24 level of each vector preparation, the transduction efficiency of HIV-3/β-gal(F) in HepG2 cells was approximately 98% lower than that of HIV-3/β-gal(G).

Cultivation of HepG2 cells on Transwell-COL cell culture membrane inserts and subsequent flow-through transduction with MLV (SV-F) pseudotypes led to significantly improved titers [18]. To increase the transduction efficiency of HIV-3/β-gal(F), HepG2 cells were cultivated on collagen-coated Transwell-COL cell culture membrane inserts, which displayed an estimated porosity of 50%. The cells were transduced either with the static method as described above or with the flow-through method [19]. The flow-through method increased the transduction efficiency of HIV-3/β-gal(F) in HepG2 cells by fourfold relative to the static method. The titer of the same vector preparation increased from 6.5×10^3 to 2.4×10^4 TU/ml. We also observed a similar increase using the flow-through transduction with HIV-3/β-gal(G), increasing the titer from 2.6×10^5 to 5.5×10^5 TU/ml.

Preferential Transduction of Hepatocytes with HIV-3/β-gal(F) Pseudotypes

To determine if the SV-F pseudotypes transduce hepatocytes specifically, we used HIV-3/β-gal(F) to transduce HeLa cells as well as several well-differentiated human hepatoma lines, including Hep3B, Huh7, and Huh6. HIV-3/β-gal(F) was able to transduce these human hepatoma cell lines, with transduction efficiencies ranging between 1.7×10^3 TU/ml in Huh6 cells and 5.3×10^4 TU/ml in Huh7 cells (Table 2). Compared with hepatoma lines, HeLa cells could be transduced only poorly. Thus, like MLV vectors, HIV vectors can be pseudotyped with the SV-F protein, leading to preferential gene delivery into hepatoma cells. However, the vector titer needs to be increased further before practical application of this system for efficient gene delivery into liver can be achieved.

Modification of the Cytoplasmic Domain of SV-F

Interactions between the cytoplasmic domain of the HIV envelope (env) protein and the viral nucleocapsid have an important role in specific incorporation of the HIV envelope protein into the virion [20–22]. To investigate whether these interactions facilitate the incorporation of SV-F and thus increase the vector titers, we constructed pCMV-Fenv, which encoded a fusion protein containing the extracellular and transmembrane domains of SV-F and the cytoplasmic domain of HIV env.

We used HIV-3/β-gal(F) and HIV-3/β-gal(Fenv) with similar levels of p24 to transduce HepG2 cells and determine vector titers by β-gal staining. The vector titers derived from pCMV-Fenv were reduced by more than 99% to 1.2×10^2 TU/ml compared with that from pCMV-F (Table 3). Because the cytoplasmic tail of the fusion protein was derived from HIV env, this reduction in titers is most likely a result of decreased stability of the fusion protein or inefficient transportation of the fusion protein from endoplasmic reticulum to cell surface [23,24]. Thus, substitution of the cytoplasmic tail of SV-F with that of HIV env failed to improve the titer of SV-F pseudotype.

TABLE 1: Titers of HIV-3/β-gal pseudotypes

	Titer (TU/ml) in	
Vector	HT1080	HepG2
HIV-3/β-gal(G)	$1.3 \times 10^6 \pm 0.5 \times 10^6$	$2.6 \times 10^5 \pm 0.9 \times 10^5$
HIV-3/β-gal(F)	0.0	$6.5 \times 10^3 \pm 1.2 \times 10^3$

HIV-3/β-gal vectors pseudotyped with SV-F or VSV-G were generated by transient transfection using pCMV-G or pCMV-F, respectively. After activation of SV-F with acetylated trypsin, the vectors were used to transduce HT1080 cells or HepG2 cells. Forty-eight hours after transduction, cells were stained for β-gal expression and the titers were determined by counting the blue cells. All experiments were carried out at least three times using independently generated vector preparations. Each titer represents the average of at least three experiments ± standard deviation.

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TABLE 2: Titers of the HIV-3/β-gal(F) pseudotype in different cell lines

Cell line	Titer (TU/ml)
HeLa	13.1 ± 5.7
Hep3B	$3.4 \times 10^4 \pm 0.9 \times 10^4$
Huh6	$1.7 \times 10^3 \pm 0.7 \times 10^3$
Huh7	$5.3 \times 10^4 \pm 0.8 \times 10^4$

HIV-3/β-gal (F) was generated as described in Table 1 and activated with acetylated trypsin. Forty-eight hours after transduction, titers were determined as described in Table 1. All experiments were carried out at least three times. Each titer represents the average of at least three experiments ± standard deviation.

Several previous studies demonstrate that the incorporation of heterologous envelope proteins into HIV or MLV particles frequently depended on the removal of part or all of the cytoplasmic domains from these proteins [25–27]. This prompted us to investigate whether shortening of the cytoplasmic domain of SV-F could enhance its incorporation into HIV particles. Short cytoplasmic tails are less likely to cause steric hindrance and to interfere with the particle formation. Thus, we generated pCMV-Fdel, encoding a truncated SV-F with only 2 amino acids out of 42 amino acids left in the cytoplasmic domain. The vector titers derived from pCMV-Fdel in HepG2 cells increased approximately threefold relative to those from pCMV-F (Table 3), suggesting that the long cytoplasmic domain of SV-F interfered with efficient incorporation of the SV-F protein into HIV particles.

Physical Concentration and Stability of SV-Fdel Pseudotyped Vectors

Vectors pseudotyped with VSV-G can be concentrated to extremely high titers by ultracentrifugation [28]. We tested whether SV-Fdel pseudotyped vectors could also sustain the centrifugation force and be concentrated similarly. HIV-3/β-gal(F) was activated by acetylated trypsin treatment either before or after concentration by ultracentrifugation at 19,500 rpm for 2.5 hours, followed by transduction of HepG2 cells. The efficiency of recovery for the vector activated before ultracentrifugation (F_1-F_2) was approximately 20–25% and the vector titers increased 16- to 20-fold, from 2.1 to 4.3×10^4 TU/ml to 3.4 to 8.6×10^5 TU/ml (Table 4). The efficiency of recovery increased to 44–56% if the vector was concentrated first before activation (F_0). In this case, the vector titers increased 36- to 44-fold, from 2.1 to 4.3×10^4 TU/ml, to 7.5×10^5 to 1.9×10^6 TU/ml. Thus, the SV-F_0 precursor seems to be more stable than its cleaved subunits F_1 and F_2 to sustain the centrifugation force when incorporated into HIV particles. These results clearly suggest that, like VSV-G, the SV-F protein is stable in an HIV particle and can be concentrated to high titers.

To investigate the stability of SV-F pseudotyped vectors against multiple freeze/thaw cycles, we generated SV-F pseudotypes by transient transfection. The titer was determined in HepG2 immediately after activation of the virus by acetylated trypsin treatment or after one, two, and three freeze/thaw cycles. Exposure of the vector to multiple freeze/thaw cycles led to a progressive decrease in titer, with only 4% of the original virus titer remaining after three freeze/thaw cycles (Table 5). Activation of SV-F with acetylated trypsin after three freeze/thaw cycles led to similar virus titers (data not shown). Thus, the inactive form of SV-F revealed the same instability against freeze/thaw cycles as the active form. In this regard, SV-F pseudotypes seem to be less stable than VSV-G pseudotypes, which retain approximately 60% of the infectivity after six freeze/thaw cycles [28].

An Increase in HepG2 Transduction with New Generations of HIV Vectors

We tested whether improvements in the vector design could significantly increase titers in HepG2 cells. Zufferey *et al.* have shown that the insertion of the posttranscriptional regulatory element sequence from woodchuck hepatitis virus (WPRE) into either retroviral or lentiviral vectors increases the expression level of the transgene [29]. To improve gene expression in hepatocytes, we inserted a 600-bp WPRE fragment downstream of the β-gal gene in pHIV-3/β-gal to create pHIV-6/β-gal (Fig. 1). Previous reports [30,31] demonstrated that several human cell types were transduced with higher efficiency when the central polypurine tract (cPPT) from HIV-1 was included in the vector. This sequence facilitates the nuclear transport of the preintegration complex [31]. To determine whether this sequence improves the transduction efficiency of hepatocytes, we inserted a 190-bp cPPT-containing fragment immediately upstream of the internal CMV promoter in pHIV-6/β-gal to create pHIV-7/β-gal (Fig. 1).

To compare the transduction efficiencies of HIV-3/β-gal, HIV-6/β-gal, and HIV-7/β-gal, we generated VSV-G and SV-F pseudotypes by transient transfection, and the titers were determined on HT1080 (for VSV-G pseudotypes) and

TABLE 3: Titers of HIV-3/β-gal pseudotyped with modified SV-F proteins

	Titer (TU/ml)	
Envelope protein expression plasmid	HT1080	HepG2
pCMV-G	$1.3 \times 10^6 \pm 0.2 \times 10^6$	$5.9 \times 10^5 \pm 1.0 \times 10^5$
pCMV-F	0.0	$2.1 \times 10^4 \pm 0.5 \times 10^4$
pCMV-Fenv	0.0	$1.2 \times 10^2 \pm 0.8 \times 10^2$
pCMV-Fdel	0.0	$6.3 \times 10^4 \pm 0.7 \times 10^4$

HIV-3/β-gal pseudotypes were generated as described in Table 1. The p24 level of each vector preparation was determined and similar amounts of p24 were used for static transduction of HT1080 cells and flow-through transduction of HepG2 cells. Forty-eight hours after transduction, titers were determined as described in Table 1. All experiments were carried out at least three times using independently generated vector preparations. Each titer represents the average of at least three experiments ± standard deviation.

TABLE 4: Concentration of HIV-3/β-gal(F) by ultracentrifugation

Envelope protein	Experiment no.	Titer (TU/ml) pre-conc.	Titer (TU/ml) post-conc.	Fold of conc.	Total virus (TU) pre-conc.	Total virus (TU) post-conc.	Recovery rate
F_1-F_2	1	2.1×10^4	3.4×10^5	16	1.7×10^6	3.4×10^5	20%
	2	4.3×10^4	8.6×10^5	20	3.4×10^6	8.6×10^5	25%
F_0	1						
	2	2.1×10^4	7.5×10^5	36	1.7×10^6	7.5×10^5	44%
		4.3×10^4	1.9×10^6	44	3.4×10^6	1.9×10^6	56%

HIV-3/β-gal(F) vectors were generated by transient transfection. Supernatants of transfected 293T cells were collected for three consecutive days and pooled. Half of the supernatant was activated with acetylated trypsin and concentrated by ultracentrifugation (F_1-F_2). The other half of the supernatant was activated after ultracentrifugation (F_0). The titer was determined after flow-through transduction of HepG2 cells by β-gal staining as described in Table 1. The results of two experiments using independently generated vector preparations were presented.

HepG2 cells (for SV-F pseudotypes). The presence of the WPRE sequence and the cPPT sequence had very little effect on the transduction of HT1080 cells (Table 6). However, in HIV-6/β-gal(G) transduced cells, the β-gal expression increased by twofold compared with HIV-3/β-gal(G) transduced cells. The presence of both the WPRE and the cPPT sequence in HIV-7/β-gal(G) resulted in a fourfold increase in β-gal expression. In HepG2 cells, the transduction efficiency for HIV-6/β-gal(F) increased 50% when compared with HIV-3/β-gal(F). The presence of both the WPRE and the cPPT sequences in HIV-7/β-gal(F) resulted in an approximately fourfold increase in vector titers when compared with HIV-3/β-gal(F) (Figs. 2A–2C). Consistent with the increase in vector titers, expression of the β-gal gene in the transduced HepG2 cells also increased. The β-gal expression in HIV-6/β-gal(F) and HIV-7/β-gal(F) transduced HepG2 cells was approximately 5- and 20-fold higher than that in HIV-3/β-gal(F) transduced cells, respectively (Table 6). The larger increase in the β-gal expression relative to the vector titers probably reflects the ability of the WPRE sequence to enhance gene expression in transduced cells. These results suggest that the combined effect of the WPRE and the cPPT sequences led to improved transduction efficiency and gene expression of SV-F pseudotyped HIV vectors in HepG2 cells. The difference in the ability of the cPPT sequence to improve titers in HT1080 and HepG2 cells suggests that its effect is not universal and is dependent on the cell type.

To determine the maximum titer of SV-F pseudotypes achievable on HepG2 cells, we generated HIV-7/β-gal vectors pseudotyped with SV-F by transient transfection using pCMV-Fdel containing the truncated SV-F gene. The culture supernatant was concentrated by ultracentrifugation and HepG2 cells were transduced using the flow-through method. HIV-7/β-gal was concentrated to 5.8×10^6 TU/ml with a recovery rate of 44%. This result shows that high-titer SV-F pseudotyped HIV vectors can be obtained. With the procedures described in this study, we increased the titer of SV-F pseudotype approximately 900-fold. With further concentration steps, the vector should ultimately be suitable for gene delivery into liver *in vivo*.

Transduction of Primary Human Cells

To test the transduction efficiency of the SV-F pseudotypes in primary human cells, we transduced primary human endothelial cells (HUVEC) and primary human hepatocytes with similar amounts of HIV7/β-gal(F) and HIV7/β-gal(G). HIV7/β-gal(F) transduced HUVEC cells very poorly with a transduction efficiency of 26.1 ± 11.3 TU/ml (Fig. 2E), whereas HIV7/β-gal(G) transduced HUVEC cells with an efficiency of $8.3 \times 10^6 \pm 0.9 \times 10^6$ TU/ml (Fig. 2D). In contrast, both vectors transduced primary human hepatocytes with similar efficiencies (Figs. 2F and 2G). However, precise estimation of the transduction efficiency in primary human hepatocytes is difficult due to large aggregates of hepatocytes under the culture conditions. Nevertheless, these results show that HIV7/β-gal(F) was able to transduce primary human hepatocytes efficiently compared with HIV7/β-gal(G).

Discussion

The development of vectors targeting specific cell types in order to reduce potential immune responses to the transgene product or the vector itself poses a major challenge facing gene therapy. The high affinity of SV-F for the hepatic ASGP-R and its ability to mediate the fusion between viral envelopes and the cell membrane are prerequisite for hepatocyte-targeted gene delivery. Our

TABLE 5: Stability of HIV-3/β-gal(F) pseudotypes

Freeze/thaw cycle	Titer (TU/ml)	% virus remaining
0	$8.4 \times 10^4 \pm 0.7$	–
1	$4.6 \times 10^4 \pm 0.3$	55%
2	$1.6 \times 10^4 \pm 0.5$	20%
3	$3.0 \times 10^3 \pm 0.7$	4%

HIV-3/β-gal(F) pseudotypes were generated by transient transfection and used for flow-through transduction of HepG2 cells immediately after activation with acetylated trypsin and after multiple freeze/thaw cycles. Titers were determined by β-gal staining 2 days after transduction. All experiments were carried out at least three times. Each titer represents the average of at least three experiments ± standard deviation.

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TABLE 6: Transduction efficiency of different HIV vectors in HT1080 and HepG2 cells

Vector	HT1080		HepG2	
	Titer (TU/ml)	β-gal activity (U/mg protein extract/ng p24)	Titer (TU/ml)	β-gal activity (U/mg protein extract/ng p24)
HIV-3/β-gal	$1.4 \times 10^6 \pm 0.1 \times 10^6$	64.3 ± 5.1	$2.3 \times 10^4 \pm 0.3 \times 10^4$	5.3 ± 1.2
HIV-6/β-gal	$1.7 \times 10^6 \pm 0.1 \times 10^6$	140.4 ± 14.4	$3.6 \times 10^4 \pm 0.3 \times 10^4$	28.3 ± 7.1
HIV-7/β-gal	$1.9 \times 10^6 \pm 0.2 \times 10^6$	259.1 ± 15.4	$1.0 \times 10^5 \pm 0.5 \times 10^5$	123.1 ± 10.1

HIV vectors pseudotyed with VSV-G and SV-F were generated by transient transfection and used for transduction of HT1080 (G) and HepG2 (F) cells. The titer was determined by β-gal staining 2 days after transduction. To compare the level of β-gal expression, cell extracts were prepared by four freeze-thaw cycles. The β-gal activity in the cell extract was determined and then normalized to the protein concentration of the extract and the amount of p24 used for transduction. All experiments were carried out at least three times using independently generated vector preparations. Each titer or the β-gal activity represents the average of at least three experiments ± standard deviation.

results show that pseudotyping of HIV vectors with the SV-F protein led to preferential transduction of hepatocytes with no or very poor transduction efficiencies in non-liver cells.

The transduction efficiency of HIV(SV-F) pseudotypes in HepG2 cells, however, was approximately 98% lower than that of HIV(G) pseudotypes. This decrease in the transduction efficiency may be due to the abundance of ASGP-R in HepG2 cells or the SV-F protein incorporated into the vector. It could also depend on the interaction between the SV-F protein and the ASGP-R. Inefficient release of the internalized vector from the endosome compartment may also account for the relatively lower transduction efficiency. In this study, we took several approaches to try to improve the vector titer. Flow-through transduction increased the titer in HepG2 cells by fourfold. This is consistent with a previous report [20] demonstrating that flow-through transduction led to a ninefold increase of MLV (SV-F) titers in HepG2 cells. Because hepatocytes such as HepG2 cells are known to grow in a polarized manner in culture [32–35] with most of the cell-surface ASGP-R molecules expressed on the basolateral site, ASGP-R molecules may not be accessible to the HIV-3/β-gal(SV-F) particles applied from the top of the HepG2 monolayer. Thus, improved access of SV-F to its cell surface receptor may explain the increased transduction efficiency. A similar increase using the flow-through transduction with HIV-3/β-gal(G) was also observed, consistent with basolateral distribution of the VSV-G receptor in polarized cells [19]. This hypothesis, however, cannot exclude the possibility that the improved transduction efficiency may be due to the method used for vector application that facilitates the diffusion of the virus particles in the culture medium, thereby increasing the efficiency of virus/cell interactions.

Shortening of the cytoplasmic domain of SV-F led to a threefold increase in vector titers. This result is consistent with previous reports demonstrating that the incorporation of heterologous envelope proteins with long cytoplasmic domains into HIV or MLV particles depended on the removal of part or all of the cytoplasmic domains [25–27]. Although the 42-amino-acid cytoplasmic domain from SV-F is relatively short compared with HIV env, which contains 150 amino acids, it can probably cause steric hindrance and interfere with efficient incorporation of SV-F into the HIV particle. Shortening of the cytoplasmic tail to two amino acids most likely removed this steric hindrance, leading to more efficient SV-F incorporation into the HIV particle and an increase in the vector titer.

Gene delivery experiments *in vivo* require the infection of a large number of cells, thus demanding high-titer vector preparations. Therefore, the ability to withstand the forces encountered during vector concentration by ultracentrifugation is an important property of HIV (SV-F) pseudotypes. The recovery rates for SV-F pseudotypes ranged from 44% to 56% after one step of ultracentrifugation. Thus, SV-F pseudotypes were less stable than VSV-G pseudotypes, which can be concentrated by ultracentrifugation with recovery rates of over 90% [28]. However, recovery rates obtained for SV-F pseudotypes were still 50 times higher than those obtained for other envelope proteins such as the MoMVL env [28]. SV-F pseudotypes also seem to be less stable than VSV-G pseudotypes in freeze/thaw cycles [28]. These instabilities may be due to the structure of SV-F, which comprises two polypeptides linked by a labile disulfide bond. Loss of the extracellular domain is believed to be the reason for the instability of retroviruses during ultracentrifugation [36,37].

Lentiviral vectors have been pseudotyped with a variety of different viral envelope proteins or chimeric variants including VSV-G, different MLV subtypes, and HTLV-1 [13,14,38]. The titers achieved with these envelope proteins were modest compared with VSV-G pseudotyped lentivirus vectors. However, none of these proteins could be used to target specific cell types. *In vivo* gene delivery into liver with HIV vectors pseudotyped with VSV-G resulted in the transduction of cells in spleen that might cause humoral or cellular immunity to the transgene product and the vector [39]. Restricting the expression of transgenes to specific cell types has been shown, leading to much weaker T-cell responses towards transgene products and prolonged transgene expression *in vivo* [8]. These

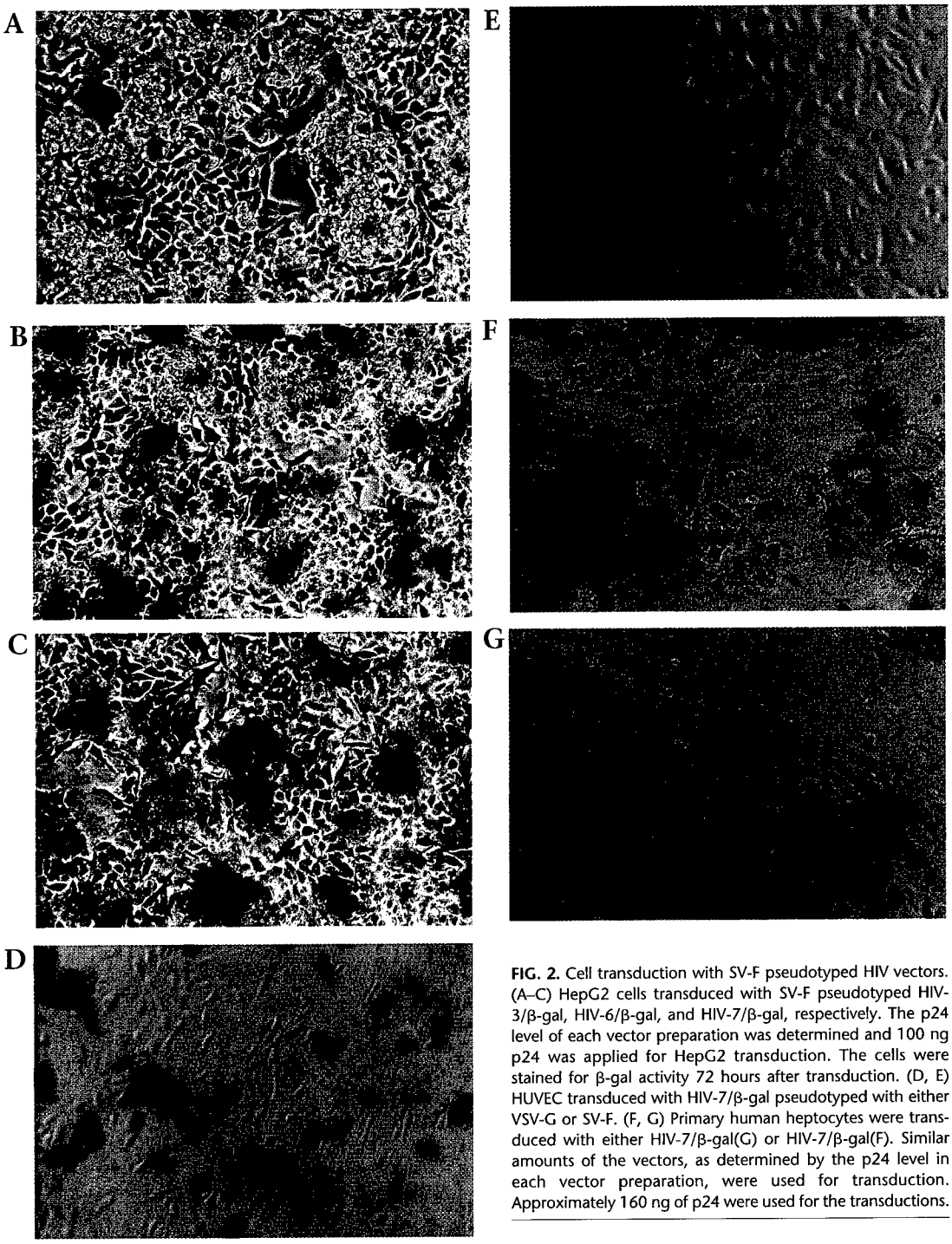

FIG. 2. Cell transduction with SV-F pseudotyped HIV vectors. (A–C) HepG2 cells transduced with SV-F pseudotyped HIV-3/β-gal, HIV-6/β-gal, and HIV-7/β-gal, respectively. The p24 level of each vector preparation was determined and 100 ng p24 was applied for HepG2 transduction. The cells were stained for β-gal activity 72 hours after transduction. (D, E) HUVEC transduced with HIV-7/β-gal pseudotyped with either VSV-G or SV-F. (F, G) Primary human heptocytes were transduced with either HIV-7/β-gal(G) or HIV-7/β-gal(F). Similar amounts of the vectors, as determined by the p24 level in each vector preparation, were used for transduction. Approximately 160 ng of p24 were used for the transductions.

results emphasize the importance of restricted transgene expression in order to lower the risk of inducing immune responses. SV-F has been incorporated into F-virosomes and used for *in vivo* gene delivery experiments targeting mouse hepatocytes. It did not elicit significant humoral immune responses in the animals [40]. Our data suggest that pseudotyping with the SV-F protein has the potential to confer hepatocyte specificity on lentiviral vectors. The use of SV-F as envelope protein for HIV vectors should restrict the transduction predominantly to hepatocytes and thus minimize potential immune responses to the transgene product or the vector itself.

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MATERIAL AND METHODS

Plasmid construction. Plasmid pCMV-F was constructed by replacing the VSV-G gene in pCMV-G with a 1.8-kb *Eco*RI/*Xho*I fragment containing the SV-F gene isolated from pcDNA3-F [18]. To create an SV-F mutant with a shortened cytoplasmic domain, a 750-bp fragment encoding the transmembrane domain and two amino acids of the cytoplasmic domain of SV-F was amplified by PCR using plasmid pCMV-F as template. The sequences of the two PCR primers used were 5′-TGTGGATCTAGAGAGATACATGGTTACCCT-3′ and 5′-CCGCTCGAGTTAGAGTCTATAAAGCA-3′.

To create plasmid pCMV-Fdel, the PCR product was digested with *Bst*EII/*Xho*I and cloned into pCMV-F, replacing a 900-bp fragment coding for the transmembrane domain and the full-length cytoplasmic domain of SV-F.

To generate plasmid pCMV-Fenv coding for a fusion protein containing the extracellular and transmembrane domains of SV-F and the cytoplasmic domain of HIV env, two DNA fragments were PCR amplified. A 750-bp fragment encoding the transmembrane domain and the N-terminal part of the cytoplasmic domain of HIV *env* was amplified using pCMV-F as template. The sequences of the two PCR primers used were 5′-TGTGGATCTAGAGAGATACATGGTTACCCT-3′ and 5′-TCCCTGCCTAACTCTATAAAGCACGATGACGATCA-3′.

A 450-bp DNA fragment coding for the C-terminal part of the transmembrane domain of SV-F and the cytoplasmic domain of HIV *env* was generated using pCMV-HIV-1 [41] as a template. The sequences of the two PCR primers used were 5′-TGATCGTCATCGTGCTTTATAGAGTTAGGCAGGGA-3′ and 5′-CCGCTCGAGTTATAGCAAAATCCTTTCCAA-3′.

To create pCMV-Fenv, both PCR products were purified, mixed, and used for primer extension. The resulting 1.2-kb PCR product was cut with *Bst*EII/*Xho*I and cloned into pCMV-F, replacing a 900-bp fragment encoding the wild-type transmembrane and cytoplasmic domains of SV-F.

The plasmid pHIV-3 has been described [42] (Fig. 1). To generate pHIV-6, a 600-bp *Bam*HI/*Sal*I fragment containing the WPRE was cloned into pHIV-3 downstream of the unique *Bam*HI site (Fig. 1). To generate pHIV-7, a 190-bp *Bgl*II/*Bam*HI fragment containing the central polypurine tract (cPPT) from HIV-1 was amplified from pCMV-HIV-1 [41] by PCR. The PCR product was cloned into pHIV-6/β-gal between RRE and the unique *Bam*HI site (Fig. 1). To create pHIV-3/β-gal, pHIV-6/β-gal, and pHIV-7/β-gal, plasmid pC-*lacZ* containing the β-galactosidase gene was digested with *Not*I/*Sma*I. A 3.8-kb fragment containing the CMV promoter and the β-gal gene was cloned in the unique *Bam*HI site in pHIV-3, pHIV-6, and pHIV-7, respectively.

Cell culture. HT1080, 293T, HepG2, Hep3B, Huh6, Huh7, and HeLa cells were maintained in high glucose (4.5 g/l) Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, and 100 mg/l gentamycin. HepG2, Hep3B, Huh6, and Huh7 were derived from human hepatocellular carcinomas. Primary human endothelial cells (HUVEC) and primary human hepatocytes were purchased from Clonetics, MD. HUVEC cells were maintained in EBM-2 medium (Clonetics). The hepatocytes were maintained in HMM medium (Clonetics).

Vector production and cell transduction. To produce infectious vectors, 4×10^6 293T cells were cotransfected with 10 μg pcDNA3-F [18], 10 μg pCMV-HIV-1 [41], and 20 μg pHIV-3/β-gal (Fig. 1) by calcium phosphate co-precipitation [43]. Plasmid pcDNA3-F contains the SV-F gene controlled by the immediate early (IE) gene promoter of cytomegalovirus (CMV). Plasmid pCMV-HIV-1 contains all HIV-1 genes except the *env* gene controlled by the CMV promoter. The HIV vector, pHIV-3/β-gal, contains the β-gal gene controlled by the CMV promoter. While Tat was expressed from pCMV-HIV-1, generation of the HIV vectors was Tat independent due to a fusion between the CMV IE enhancer and the HIV5′LTR in which the HIV sequence upstream of the TATA box was replaced by the CMV enhancer (Fig. 1). The HIV promoter and enhancer sequences in the 3′ LTR were removed, leading to the generation of self-inactivating (SIN) HIV vectors (Fig. 1) [44]. As a control, the same HIV vector pseudotyped with the vesicular stomatitis virus envelope glycoprotein (VSV-G) was similarly generated with VSV-G expression plasmid pCMV-G [45]. Six hours after transfection, the culture medium was replaced with fresh serum-free medium containing 1% Ultraculture (Biowhittaker, Walkersville, MD), because serum-free conditions were required to convert the $SV\text{-}F_0$ precursor into the fusion-active $F_1\text{-}F_2$ form by acetylated trypsin treatment [15]. The supernatant was harvested 24 hours after transfection and the virus was activated by acetylated trypsin as described [18]. To determine the vector titers, 10^5 HepG2 cells or HT1080 cells were seeded in a 6-well plate in the presence of 4 μg Polybrene/ml. The cells were transduced for 5 hours with various dilutions of the vector. The cells were stained for β-gal activity 72 hours after transduction. The p24 level of the vector preparations was determined using the Coulter HIV-1 p24 antigen assay kit (Beckman Coulter, CA).

Flow-through transduction. HepG2 cells were plated out and cultivated on collagen-coated Transwell-COL cell culture membrane inserts (Corning, NY), which displayed an estimated porosity of 50%. For flow-through transduction, the medium was removed from the outer chamber and virus-containing medium was applied to the insert, inducing flow-through by gravity. The medium was reapplied to the insert every 45 minutes. After 5 hours, the medium was replaced with fresh medium. The transduction efficiency was determined by β-gal staining 48 hours after transduction.

The β-gal assay. Cell extracts were prepared 3 days after transduction. The cells were resuspended in 100 μl of 250 mM Tris-HCl, pH 7.8, and subjected to four freeze-thaw cycles. Cell debris was removed by centrifugation and the supernatant was used for the β-gal assay. The β-gal activity in the cell extracts was determined by adding 50 μl of the extract to 450 μl of the β-gal buffer (0.05 M Tris-HCl, pH 7.5/0.1 M NaCl/0.01 M $MgCl_2$) containing 0.75 mg/ml *o*-nitrophenyl-β-D-galactopyranoside (ONPG). The samples were incubated at 37°C for 10 minutes and the reaction was terminated by adding 500 μl of 1 M Na_2CO_3. The β-gal activity was determined by measuring the OD_{420} with visible light. The units of active β-gal were determined from a standard curve of the β-gal activity using purified β-gal proteins (Sigma, MO).

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1983 - 1986	Postdoctoral Fellowship, University of California at San Diego

Teaching Experience:

1976 - 1977	Teaching Assistant for laboratory courses in Biochemistry and Microbiology, Department of Biology, Fu Jen Catholic University, Taipei, Taiwan
1979 - 1983	Teaching Assistant for the laboratory course in Microbiology, Department of Molecular Biology, University of Texas at Dallas, Dallas, Texas
1994 - 1996	Instructor for the course of Molecular and Cellular Biology in the Graduate Program, City of Hope, Duarte, California
2001-present	Instructor for Breast Cancer Biology in the Graduate Program, City of Hope, Duarte, California

Scientific Committees:

Ad hoc Reviewer for NIDDK study section (1991)

Ad hoc Reviewer for Wellcome Trust Foundation (1991)

Ad hoc Reviewer for NIDDK study section (1992)

Ad hoc Reviewer for NIDDK study section (1994)

Reviewer for NIH SBIR Special Study Section (2000- 2001)

Ad hoc Reviewer for NIH Recombinant DNA Advisory Committee (2001)

Grant Support:

Project Title: Hematopoietic cell transplantation for hematologic malignancy

P01 CA30206	NIH/NCI	4/1/00 - 3/31/05
Role: Co-PI (PI: S. Forman)	20 % effort	

Project Title: Transduction of ribozymes into CD34+ stem cell in AIDS
P01 A146030 NIH 9/1/99-8/31/03
Role: Project Leader (PI: J. Zaia) 30% effort

Project Title: Anti-angiogenic gene therapy of prostate cancer with lentiviral vectors
New Investigator Award Prostate Cancer Research Program, US Army
03/01/01-02/28/04
Role: PI 10% effort

Patent granted

Patent No. 6,133,027
Title: Inducible expression system
Inventors: **Yee; Jiing-Kuan**; Friedmann; Theodore; Chen; Shin-Tai
Assignee: City of Hope (Duarte, CA); The Regents of the University of California (Oakland, CA)

Patent No. 5,817,491
Title: VSV G pseudotyped retroviral vectors
Inventors: **Yee; Jiing-Kuan**; Emi; Nobuhiko; Friedmann; Theodore; Jolly; Douglas J.; Barber; Jack R.
Assignee: The Regents of the University of California (Oakland, CA); Chiron Viagene, Inc. (Emeryville, CA)

Patent No. 5,739,018
Title: Packaging cell lines for pseudotyped retroviral vectors
Inventors: Miyanohara; Atsushi; **Yee; Jiing-Kuan**; Chen; Shin-Tai; Prussak; Charles Edward; Friedmann; Theodore
Assignee: The Regents of the University of California (Oakland, CA); City of Hope (Duarte, CA)

Patent No. 5,670,354
Title: Use of VSV-G pseudotyped vectors for transfer of genes into embryos
Inventors: Burns; Jane C.; **Yee; Jiing-Kuan**; Friedmann; Theodore
Assignee: The Regents of the University of California (Oakland, CA)

Patent No. 5,512,421
Title: Generation, concentration and efficient transfer of VSV-G pseudotyped retroviral vectors
Inventors: Burns; Jane C.; **Yee; Jiing-Kuan**; Friedmann; Theodore
Assignee: The Regents of the University of California (Oakland, CA)

Publications:

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2. **Yee, J.-K.** and Marsh, R.C. "Locations of Bacteriophage T4 Origins of Replication." **Journal of Virology 54**, 271-277 (1985).

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5. **Yee, J.-K.**, Jolly, D.J., Moores, J.C., Respess, J.G. and Friedmann, T. "Gene Expression from a Transcriptionally Disabled Retroviral Vector Containing an Internal Promoter," **Cold Spring Harbor Symposia on Quantitative Biology**, pp.1021-1026 (1986).

6. Gruber, H.E., Finley, K.D., Luchtman, L.A., Hershberg, R.M., Katzman, S.S., Laikind, P.K., Meyers, E.N., Seegmiller, J.E., Friedmann, T. and **Yee, J.-K.** "Insertion of hypoxanthine phosphoribosyltransferase cDNA into human bone marrow cells by a retrovirus." **Adv. Exp. Med. Biol. 195**, 171-175 (1986).

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Book Chapter

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3. **Yee, J.-K.** "Retroviral vectors" in **The Development of Human Gene Therapy**, Friedmann, T., ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, Chapter 2, (1998).

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